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Optimization of a multi-gene HIV-1 recombinant subtype CRF02_AG DNA vaccine for expression of multiple immunogenic forms☆

Dennis Ellenberger,[a,*] Bin Li,[a] James Smith,[b] Hong Yi,[c] Thomas Folks,[a] Harriet Robinson,[b] and Salvatore Butera[a]

[a] *HIV and Retrovirology Branch, Division of AIDS, STD, and TB Laboratory Research, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Public Health Service, U.S. Department of Health and Human Services, Atlanta, GA 30333, USA*
[b] *Yerkes Regional Primate Research Center, Emory University, Atlanta, GA 30322, USA*
[c] *Emory Neurology Microscopy Core Laboratory, Atlanta, GA 30322, USA*

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Abstract

We developed an AIDS vaccine for Western and West-Central Africa based on a DNA plasmid vector expressing HIV-1 recombinant subtype CRF02_AG *gag*, *pol*, and *env* genes. To optimize the production of noninfectious HIV-like particles (VLPs) and potentially improve the effectiveness of the vaccine, we generated four potential vaccine constructs: the parental (IC2) and three modifications (IC25, IC48, and IC90) containing mutations within the HIV protease. While the parental construct IC2 expressed aggregates of Gag proteins, the IC25 construct resulted in the production of immature VLPs (the core comprises unprocessed Pr^{55Gag}). The remaining two constructs (IC48 and IC90) produced mature VLPs (the core comprises processed capsid p24) in addition to immature VLPs and aggregates of Gag proteins. VLPs incorporated significant levels of mature gp120 envelope glycoprotein. Importantly, the mature VLPs were fusion competent and entered coreceptor-specific target cells. The production of multiple antigenic forms, including fusion-competent VLPs, by candidate DNA vaccine constructs may provide immunologic advantages for induction of protective cellular and humoral responses against HIV-1 proteins.
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Keywords: HIV-like particles; VLP; HIV-1 DNA vaccine; Protease mutation; CRF02_AG

Introduction

The quest to identify an effective human immunodeficiency virus (HIV) vaccine has resulted in the development of many novel candidates. Because of the lack of knowledge of correlates of immune protection, many vaccine approaches and concepts are being pursued. Vaccines have been designed to raise cellular immunity to control virulent challenges and shown to prevent the development of AIDS in rhesus macaques (Amara et al., 2001; Barouch et al., 2000, 2001; Rose et al., 2001; Shiver et al., 2002). Vaccination with plasmid DNA alone can elicit both humoral and cellular immune responses (Tang et al., 1992; Ulmer et al., 1993; Wolff et al., 1990) that protect nonhuman primates against challenges with nonpathogenic AIDS viruses (Boyer et al., 1997; Letvin et al., 1997) and afford modest protection against disease from pathogenic simian immunodeficiency virus (SIV) (Egan et al., 2000; Lu et al., 1996). Another approach to raise cellular immunity is the use of DNA to prime an immune response followed by recombinant poxvirus boosters such as the modified vaccinia virus Ankara (Amara et al., 2001; Robinson et al., 2000). Including multiple HIV-1 gene regions on the same construct augments this approach, as both *gag* and *env* responses are important to protection against virus challenge (Amara et al., 2002).

One further theoretical advantage of multigene HIV-1 DNA vaccines is an opportunity to generate noninfectious HIV-like particles (VLPs). Ideally, VLPs are generated by

☆ This study was carried out under an approved protocol in accordance with guidelines set forth by the Centers for Disease Control and Prevention.

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* Corresponding author. HIV and Retrovirology Branch, Centers for Disease Control and Prevention, Mail Stop G-19, 1600 Clifton Road, Atlanta, GA 30333. Fax: +1-404-639-2919.
E-mail address: dellenberger@cdc.gov (D. Ellenberger).

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the host and presented to the immune system as multiple well-defined epitopes in a native conformation, obviating the problem of HLA restriction and effective stimulation of the MHC Class I arm of the immune response. VLPs released from transfected cells could further enhance immunogenicity by entering another round of normal target cells via fusion of the viral envelope glycoprotein (Env) with the host cell's plasma membrane (Hernandez et al., 1996). This process may allow presentation of viral antigens, in a manner analogous to that of natural HIV-1 infection. This theoretical advantage may be realized only if the released VLPs are functional with regard to fusion and entry into additional target cell populations. Furthermore, this advantage would not be available to VLP-producing HIV-1 DNA vaccines that encode only Gag or encode Gag, Env, and other viral proteins on separate DNA constructs (Barouch et al., 2000, 2002; Kang et al., 1999).

The generation of fusion-competent VLPs from cells acquiring multigene HIV type 1 (HIV-1) DNA vaccines is complicated by the dysregulation of viral gene product expression. In natural virus maturation, protease activity increases dramatically following budding from the host cell, resulting in the complete processing of the three HIV-1 precursor proteins and thus maturing the virus to its infectious form (Gottlinger et al., 1989; Peng et al., 1989). Intracellular activation of protease, instead of extracellular activation, may cause aggregation of viral proteins, limiting VLP production, and results in the appearance of immature and aberrant virus-like structures (Karacostas et al., 1993). Furthermore, a large accumulation and retention of HIV proteins within expressing cells is not a normal aspect of viral replication and may not promote processing of peptides derived from endogenous antigens within the cytoplasm of the cell and that are most effectively eliminated by cytotoxic T lymphocytes.

We undertook this study to improve the potential immunogenicity of our DNA vaccine construct. Our results show that by altering or mutating the HIV protease, we can shift the qualitative production of HIV

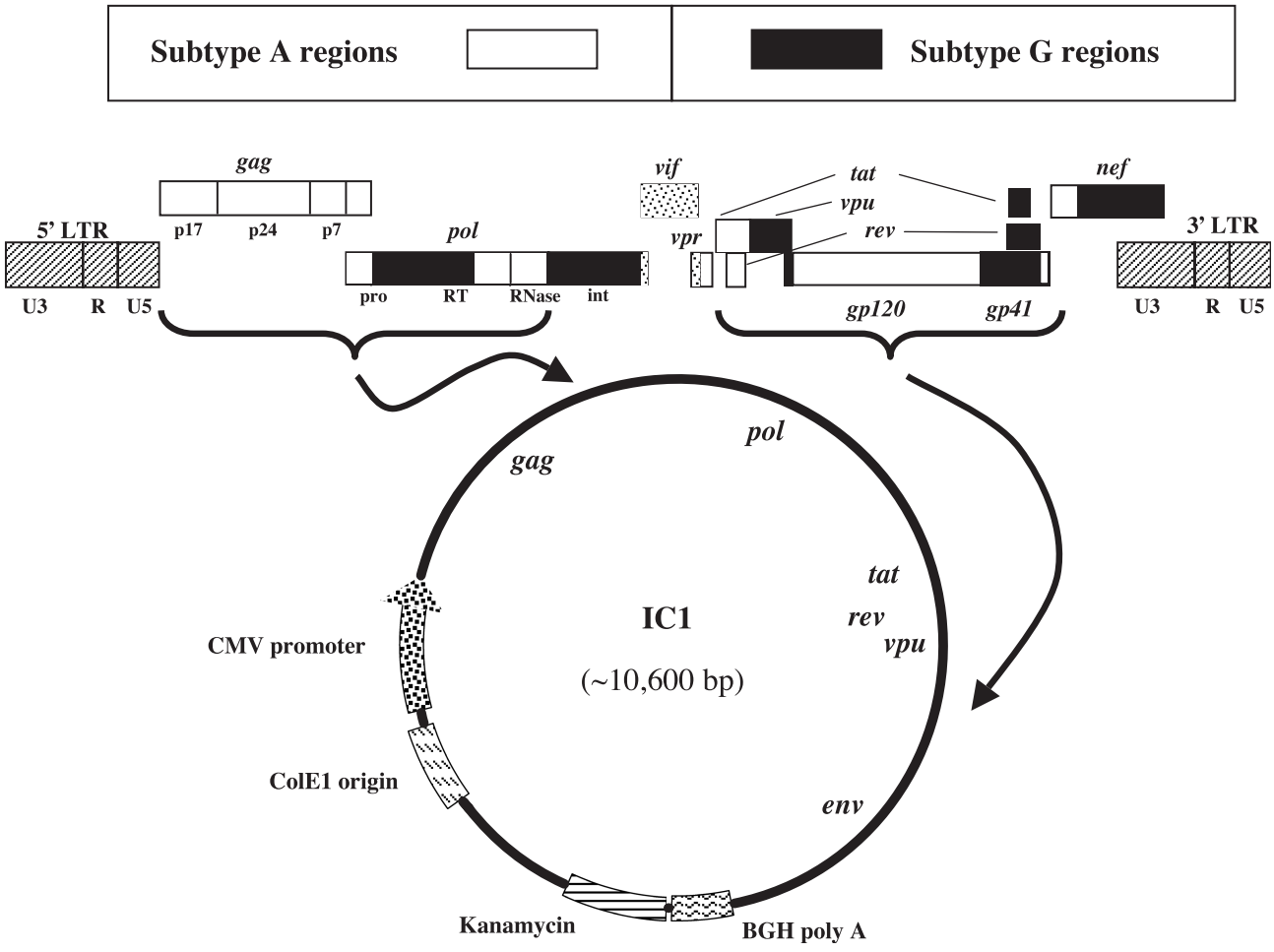

Fig. 1. Schematic representation of vaccine construct IC1. Subtypes A and G regions are identified. Stippled region of genome is unclassified subtype. LTRs are included for genomic reference. Brackets indicate genomic regions cloned into vector pGA1.

Table 1
Mutations introduced in vaccine constructs[a]

Vaccine construct	Safety mutations[b,c]	Protease mutations[d]
IC1	None	None
IC2	*gag* NC, *pol* RT	None
IC25	*gag* NC, *pol* RT	D25N
IC48	*gag* NC, *pol* RT	G48V
IC90	*gag* NC, *pol* RT	M90L

[a] For details, see Materials and methods.
[b] *Gag* P7 nucleocapsid (zinc finger 1 and 2) positions 390, 393, 411, and 414 (all C to S).
[c] *Pol* RT position D185N, W266T, and E478Q.
[d] Position of amino acid change in *pol* protease.

products. The generation of immature and mature enveloped VLPs as well as protein aggregates could provide distinct advantages to only one HIV product, providing the best level of protection by controlling HIV-1 replication and perhaps infection.

Results

Expression of vaccine construct HIV proteins

A representative CCR5 using HIV-1 CRF02_AG isolate from an incident infection in Abidjan was selected to begin construction of a multigene DNA vaccine. Using viral RNA derived from the peripheral blood of this individual, the 5′ Gag-Pol (*gag*, *protease*, and *RT*) and 3′ Env (*vpu*, *tat*, *rev*, and *env*) fragments were recombined in the pGA1 expression vector to establish the model construct, labeled Ivory Coast (IC) IC1 (Fig. 1). Two HIV genes within IC1 were then mutated by site-directed

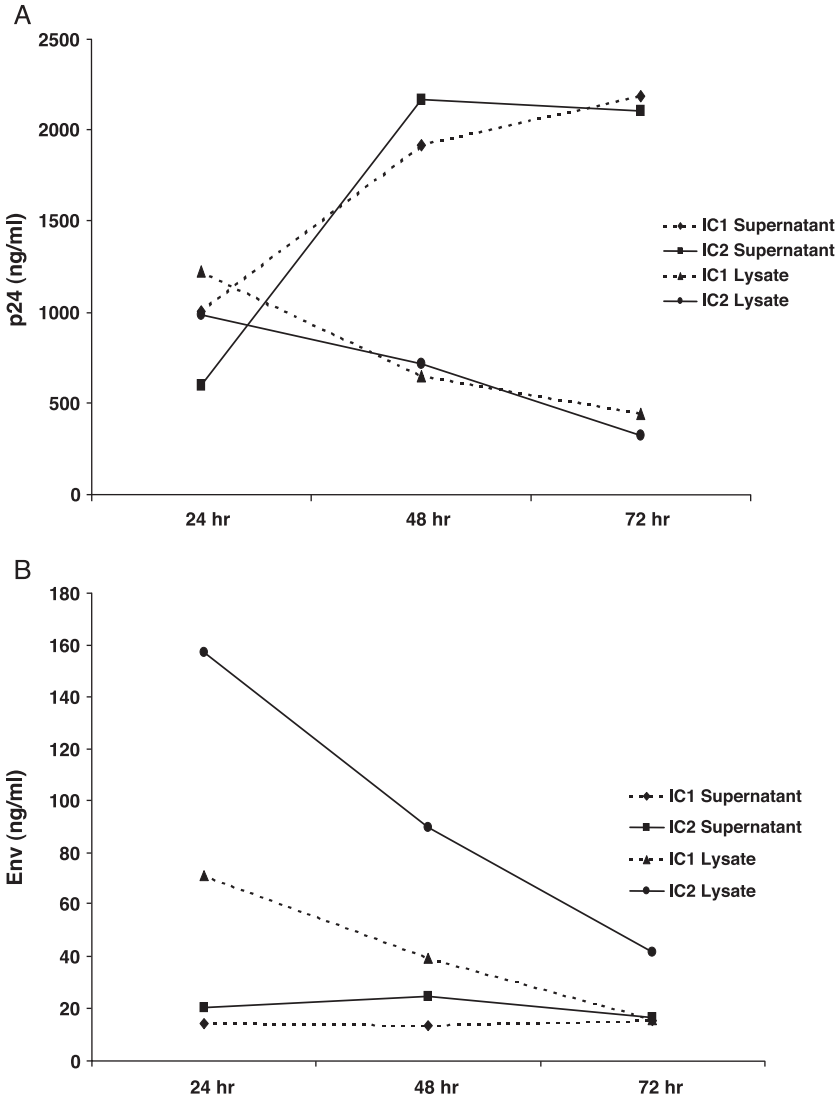

Fig. 2. HIV-1 Gag and Env expression by IC1 and IC2 vaccine constructs. (A) Gag and (B) Env expression were measured by antigen-specific ELISA.

mutagenesis to increase the vaccine's safety for potential use in humans. Mutations in *gag* NC to disable the zinc fingers and *pol* RT to inactivate viral RT activity were completed to establish the parental vaccine candidate IC2 (Table 1). The effects of these safety mutations were verified by appropriate RNA packaging and RT activity assays (data not shown). Despite the disablement of the Gag zinc fingers, processing of the Gag precursor was observed in our vaccine constructs. We did, however, observe some accumulation of Pr41, suggesting that the processing was slightly delayed, similar to findings by others (Mizuno et al, 1996).

HIV-1 protein (Gag and Env) expression for these constructs was then compared by examining cell lysates and culture supernatants by ELISA following transient transfection of 293T cells (Figs. 2A and B). HIV-1 protein expression by IC1 was greater than 1.0 μg/ml of Gag p24 and 85 ng/ml of Env in the lysate and supernatant, respectively. No reduction of Gag and Env expression was observed for IC2, because of the introduction of safety mutations, as compared with IC1. Gag production continued to increase for both constructs through 72 h; however, after 24 h, lysis of transfected cells was observed to release proteins directly into the supernatant.

Because these vaccine candidates are multigene constructs, VLP production from transfected cells was expected. To determine whether VLP formation occurred, IC2-transfected 293T cells were examined by transmission electron microscopy (Fig. 3). Surprisingly, only a limited number of VLPs (approximately 100 nm in size) were observed along with an abundance of protein aggregates. The lack of VLPs and the appearance of protein aggregates were not a result of the safety mutations, as similar findings were observed for IC1 (data not shown). The protein aggregates were presumably HIV-related proteins, as they were not observed in mock-transfected 293T cells (data not shown).

Demonstrable HIV protein expression from genetic alteration of protease activity

Because VLP assembly and release from cells depend on proper intracellular protease regulation to preserve the Gag polyprotein (Gottlinger et al., 1989; Karacostas et al., 1993; Peng et al., 1989) and may be observed upon transfection of Gag-only expression systems (Huang et al., 2001; Kang et al., 1999; Schneider et al., 1997), an HIV-1 protease inhibitor was added to the transient transfections in an attempt to improve the efficiency of VLP assembly. We treated 293T cells following IC2 transfection with saquinavir (Jacobsen et al., 1995), and as expected, the formation of Gag p24 was significantly reduced in the presence of this compound (Fig. 4A). Saquinavir-treated cultures (100 nM) contained almost exclusively p55 and p41 (>99% of the immunoreactive protein) and only trace levels of p24 (<1% of the immunoreactive protein).

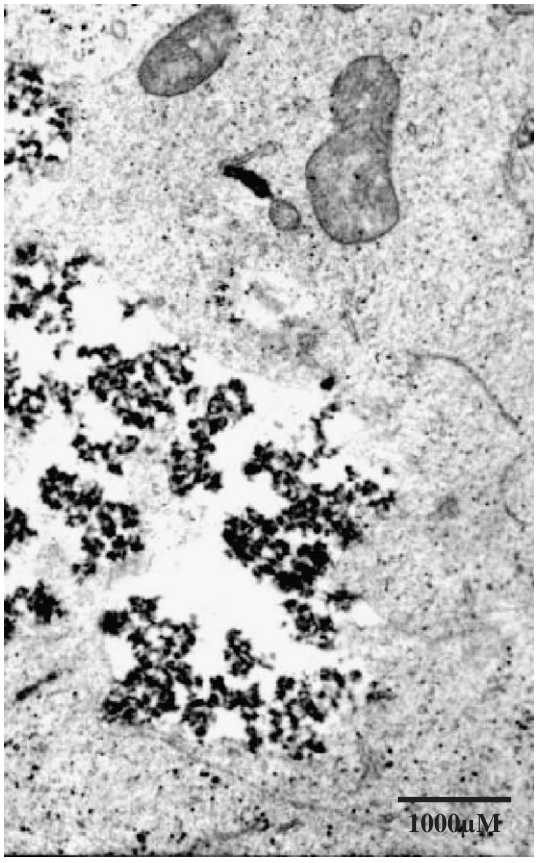

Fig. 3. Transmission electron microscopy of HIV-1-like particles in 293T cells transfected with vaccine construct IC2.

Examination of saquinavir-treated, IC2-transfected 293T cells by transmission electron microscopy demonstrated the presence of budding structures and production of morphologically immature (electron-dense shells representing the virus core and with electron-lucent centers) and uniformly sized VLPs (Fig. 4B). The number of these particles and the lack of protein aggregates were in marked contrast to IC2-transfected 293T cells not treated with protease inhibitor (Fig. 3).

Restored particle formation from genetic alteration of protease activity

The results obtained with the protease inhibitor suggested that genetic modification of protease activity might enhance production of VLPs from the vaccine constructs. Previous studies demonstrated that alteration of the 25th residue of protease, from Asp (D) to Asn (N), resulted in complete loss of protease activity (Gottlinger et al., 1989; Kohl et al., 1988; Loeb et al., 1989). Furthermore, Jacobsen et al. (1995) demonstrated that mutagenesis of protease at positions 48 (G48V) and 90 (M90L) led to less efficient enzymatic activity and delayed processing of the gag and gag-pol polyproteins. Accordingly, we created three protease mutant constructs (IC25, IC48, and

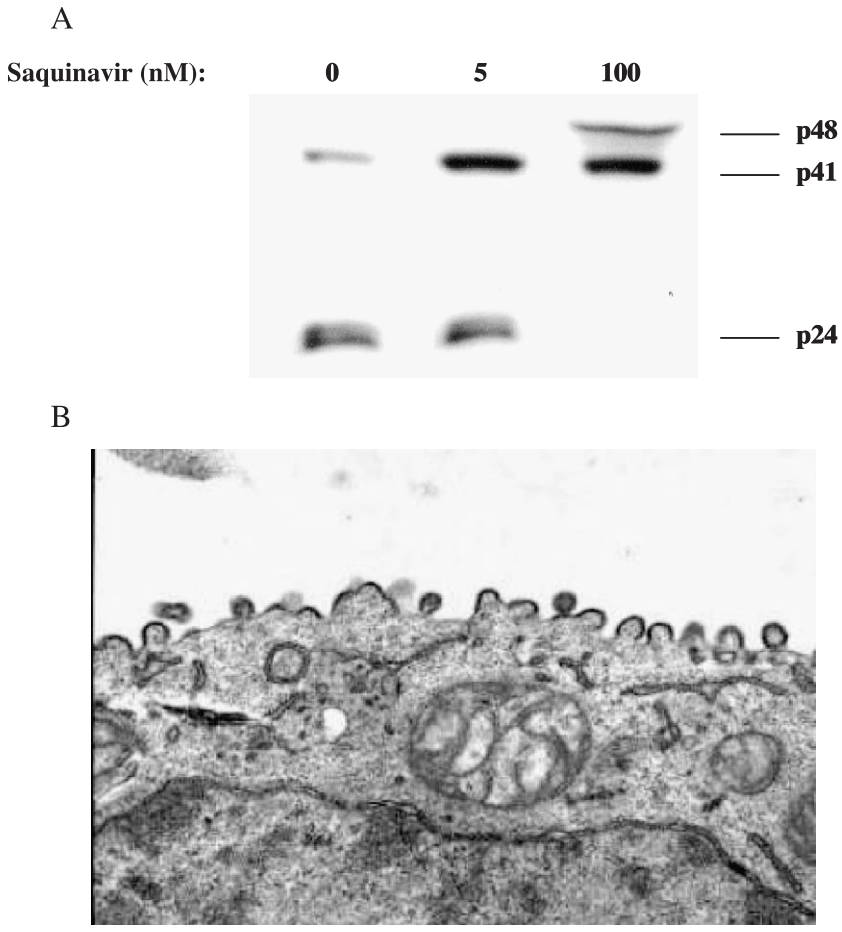

Fig. 4. Processing of Gag polyprotein in the presence or absence of saquinavir. Cells were treated with two different concentrations of saquinavir as indicated. (A) IC2-transfected 293T cell supernatants 24 h posttransfection were harvested, separated by SDS PAGE, transferred to nitrocellulose, and analyzed by immunoblotting with rabbit polyclonal anti-p24. The positions of Gag proteins are indicated on the right. (B) Transmission electron microscopy of HIV-1-like particles in 293T cells transfected with vaccine construct IC2 in the presence of 100 nM saquinavir.

IC90), making amino acid substitutions at three independent positions–25, 48, and 90—by site-directed mutagenesis (Table 1).

Gag expression was detected by antigen-capture ELISA following 293T cell transfection, and two protease mutant constructs (IC48 and IC90) appeared to express as well as IC2 (Fig. 5A). Gag expression of IC25 appeared sharply diminished as compared with IC2 and the other protease mutant constructs. However, as previously reported (Schneider et al., 1997), the detection efficiency of Gag p55 was substantially less than that of Gag p24. Quantification of total Gag protein of the IC25 supernatant by Western blot analysis (Fig. 5B), using an anti-Gag antibody with reactivity against multiple cleavage products, demonstrated that IC25 expressed Gag protein to a level equal to if not greater than the level of the other constructs (Fig. 5B). For IC25, the Gag protein was primarily p55 and other Gag polyproteins (87% of the immunoreactive protein) as expected in the absence of protease enzymatic activity. For IC2, IC48, and IC90, the majority of Gag immunoreactive protein was Gag p24 (87%, 56%, and 68%, respectively).

Production of Env protein by the various multigene vaccine constructs was also detected by ELISA (Fig. 5C). Comparable levels of total Env expression were detected for IC2, IC25, and IC48, but levels were diminished with IC90. A greater percentage of total Env protein was detected in the supernatant of all three protease mutant constructs as compared with IC2, raising the possibility of more efficient release by VLP assembly.

Detection of processed HIV proteins from sucrose-gradient-banded VLPs

To more accurately assess the assembly of VLPs, transfection culture supernatants were banded through a sucrose gradient and subsequent fractions were examined for the presence of Gag p24. Peak Gag p24 fractions were then analyzed by immunoblotting using rabbit polyclonal anti-p24 antibody (Fig. 6A). Only two Gag polyproteins, p55 and p41, were detected for IC25. However, three forms of Gag (p55, p41, and p24) were observed for IC48 and IC90, indicating cleavage of the

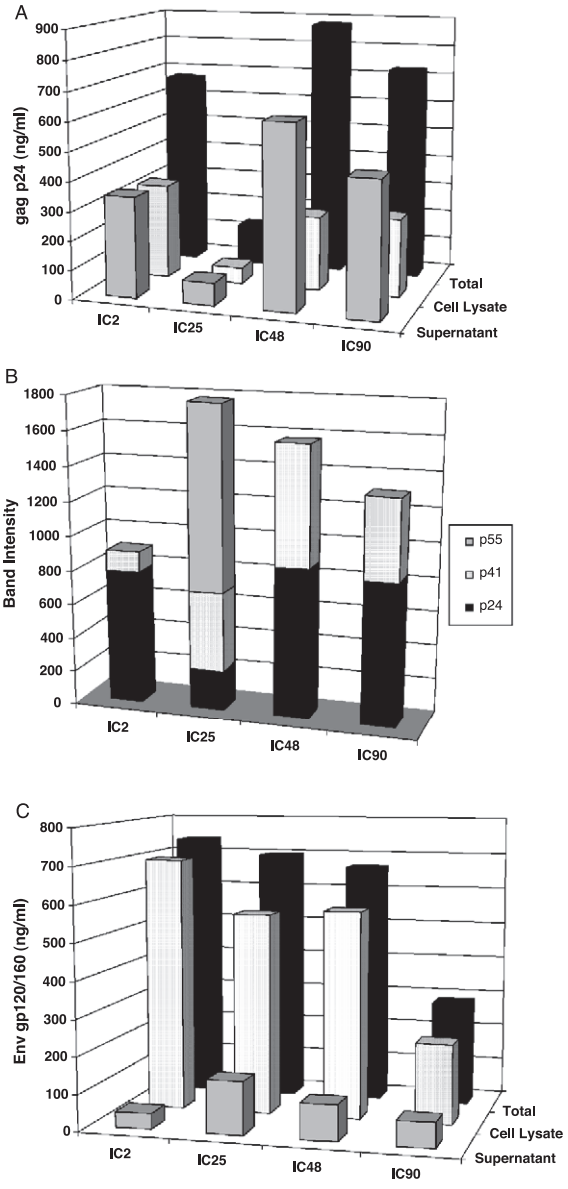

Fig. 5. HIV-1 Gag and Env expression in transfected 293T cells with various DNA vaccine constructs. (A) Gag expression in culture supernatants and cell lysates 24 h posttransfection were measured by antigen-specific ELISA. (B) Transfected cell culture supernatant Gag proteins were analyzed by immunoblotting using a rabbit polyclonal anti-p24 antibody as measured by a phosphorimager. (C) Env expression in culture supernatants and cell lysates 24 h posttransfection were measured by antigen-specific ELISA.

Gag polyprotein and maturation of VLP core structure, which included the mature core (p24) protein. The sucrose-gradient-banded VLP preparations from IC25 and IC48 also incorporated significant levels of surface HIV Env, but levels were decreased with IC90 (confirming the outcome of Env ELISA (Fig. 5C)). As anticipated for a multigene construct, upon immunoblotting of sucrose-gradient-banded VLP, only the cleaved gp120 Env glycoprotein was detected while no gp160 precursor molecules were observed (Fig. 6B).

Confirmation of mature VLP assembly from partial inactivation of protease

To further confirm that genetic inactivation (i.e. IC25) or partial inhibition (i.e. IC48) of protease activity permitted VLP assembly and possible particle maturation, cells transfected with protease-mutant vaccine constructs were examined by electron microscopy (Fig. 7). For IC25, immature VLPs were seen (Fig. 7A). These particles were uniformly sized, abundant in number, and similar in appearance (electron-dense shells and lucent centers) to those formed by expression in the presence of saquinavir (Fig. 4B). Budding structures and numerous VLPs were also observed for IC48 (Fig. 7B). But, two distinctive particle morphologies were observed: VLPs with a spherical protein shell (immature) and other particles with electron-dense centers (mature). Furthermore, the VLP with electron-dense centers had a lean spherical protein shell, suggesting cleavage of Gag p55 and condensing of the viral core, comparable to mature HIV particles. IC48-transfected cells were also observed to contain aggregates of HIV proteins (Fig. 7C), and overall, fewer VLPs were observed than with IC25-transfected cells. Although, total Gag production among these constructs was similar, Gag production by IC25 appeared to be entirely VLP based, while Gag production by IC48 was divided between multiple antigenic forms, including protein aggregates, immature VLPs, and mature VLPs.

Fusion and entry of mature HIV-like particles into target cells

To determine if VLP-incorporated Env was fusion competent and coreceptor specific and if maturation of the core would influence entry, we measured the entry of noninfectious VLPs from IC25 and IC48 into target cells. Using GHOST-4 cells stably expressing either CXCR4 or CCR5, the products from transfection with either IC25 or IC48 (engineered into the IC1 backbone to increase the level of RNA packaging) were evaluated for entry by detection of post-fusion intracellular HIV RNA (Fig. 8). Intracellular viral RNA was not detected after GHOST-4 were exposed to supernatants from mock transfections and no amplified products were observed in any samples by nested DNA PCR without first generating cDNA by a RT step (data not shown). A background level of VLP entry, presumably by endocytosis and the ability of some CCR5-using virus to enter CXCR4 cells, was observed in CXCR4-expressing cells and was similar in magnitude for both IC25 and IC48 products (Fig. 8). Similar levels of background were observed for experiments using parental GHOST-4 cells (data not shown). Entry of the non-mature VLPs produced by IC25 into CCR5-expressing cells did not score above the level observed as background. However, entry of products from IC48 transfection, including mature VLPs, into CCR5 target cells

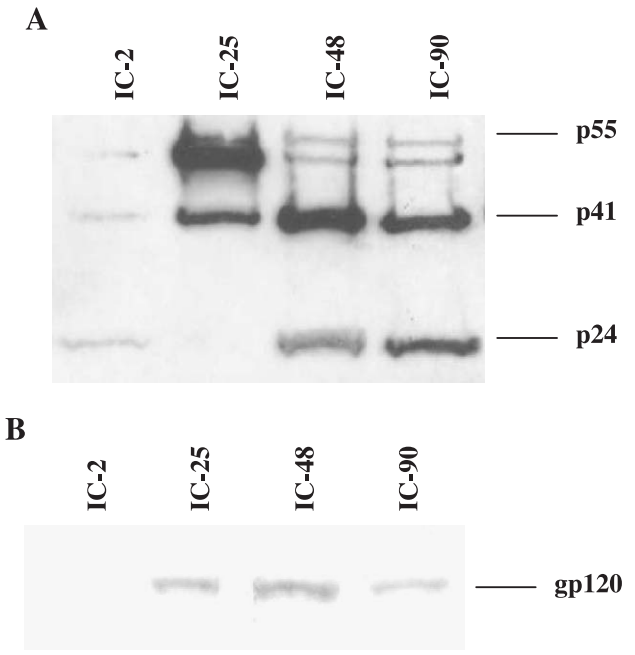

Fig. 6. Immunoblot analysis of sucrose-gradient purified HIV-1-like particles using (A) rabbit polyclonal anti-p24 and (B) goat polyclonal anti-gp160. The positions of HIV precursors and products are indicated on the right. Fraction 9 from each gradient was separated by SDS-PAGE, transferred to nitrocellulose, and analyzed by immunoblotting.

was at least 10-fold greater than background levels. These results confirm that the unique products of IC48 vaccine constructs enter cells in a manner analogous to wild-type HIV-1 fusion and may provide additional immunologic advantages.

Discussion

We describe the generation and optimization of HIV-1 DNA vaccine constructs based on the recombinant CRF02_AG subtype. The choice of isolate as the basis of these vaccine constructs was the result of extensive molecular epidemiological surveys recently conducted in Côte d'Ivorie (Ellenberger et al., 1999, 2002; Nkengasong et al., 2000). Among the potential vaccine candidates, a particular isolate from an incident HIV infection, whose Env preferentially utilized CCR5 and whose sequence closely aligned with the consensus sequence for this survey, was chosen. These HIV-1 vaccine constructs should be considered applicable to potential trials throughout broad areas of Western and West-Central Africa where the CRF02_AG subtype predominates (Andersson et al., 1999; Carr et al., 1998; Laurent et al., 2002; McCutchan et al., 1999; Montavon et al., 2000; Nkengasong et al., 2000; Peeters et al., 2000; Toure-Kane et al., 2000). Furthermore, because these constructs are subtype A in *gag* and a substantial portion of *env* (Fig. 1), they could serve as candidate vaccines in larger

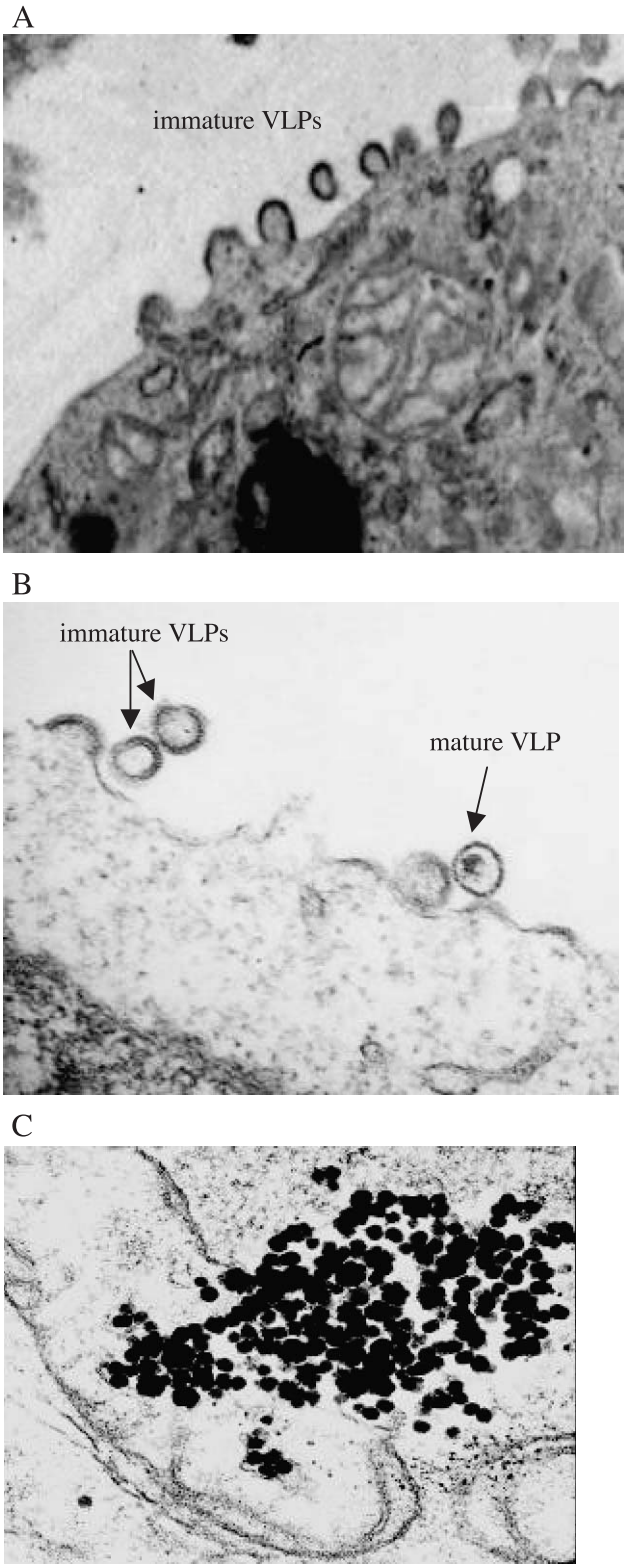

Fig. 7. Transmission electron microscopy of HIV-1-like particles in 293T cells transfected with vaccine constructs expressing HIV genes. (A) IC25, (B) IC48, and (C) IC48. Budding, extracellular immature and mature HIV-like particles are identified.

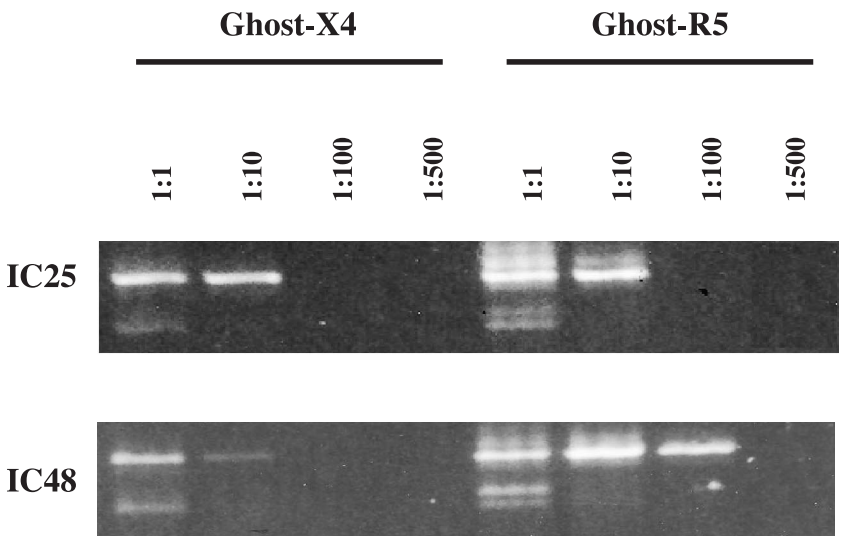

Fig. 8. Detection of viral RNA within GHOST-4-CXCR4 and -CCR5 target cells by RT-PCR. Neat culture supernatant from 293T cells, 24 h after transfection with either IC25 or IC48 was added to GHOST-4 cells for 1 h at 37°C. VLPs bound to the cell surface were removed by trypsin treatment. Following RNA extraction, viral RNA was detected by PCR, using HIV specific primers.

geographic regions where HIV-1 subtype A or other recombinant forms of subtype A exist (Ellenberger et al., 2002). These vaccine candidates might also be considered for use in geographic regions where HIV-1 subtype G circulates since subtype G comprises 26% of the vaccine, including the immunodominant region of Env gp41 and other genomic regions.

We sought to optimize expression from the DNA vaccine constructs, not only in terms of high-level production of HIV-1 proteins, but also with regard to assembly of VLPs. In addition to the provision of multiple viral gene products and potential for broad immune response, in vivo production of VLPs is another advantage of a multigene DNA vaccine approach when viral products are all expressed on a single construct. In this manner, the products from these vaccine constructs may mimic the highly effective immunization achieved with live-attenuated and live-defective virus vaccines, without the potential risk of causing infection and disease (Daniel et al., 1992; O'Neill et al., 2002) or integration. Furthermore, when the use of these DNA-based vaccines is combined with a boost immunization, such as a modified vaccinia (MVA) construct encoding the same antigens, generation of a potent and broad cellular immune response characteristic of protective DNA-MVA vaccine regimens (Amara et al., 2001) would be anticipated.

It is well established that VLPs can be generated by expression of only HIV-1 *gag* (Gheysen et al., 1989) and that single gene "*gag*-only" vaccine constructs generate VLPs following transfection of human cells (Huang et al., 2001). Although the plasmid constructs used in our study contain seven HIV-1 genes (including the *pol* protease) from an HIV-1 CRF02_AG strain, we still found it surprising that the parental IC2 vaccine construct failed to assemble VLPs to any appreciable extent. Because the viral protease is lacking in VLP-expressing *gag*-only constructs, we rationalized that aberrant intracellular activation of protease or premature cleavage of the gag polyprotein was limiting VLP assembly and budding from IC2 transfected cells. When IC2 protease activity was eliminated chemically or by direct mutagenesis of the catalytic site (IC25 construct), abundant immature VLPs with electron-dense shells and lucent centers were observed (Figs. 4B and 7A), much like those seen with *gag*-only constructs. Importantly, the introduction of protease mutations did not reduce the overall level of viral protein expression from the vaccine constructs. Therefore, strict regulation of protease function is critical for the optimal assembly of VLPs from multigene HIV-1 DNA vaccine constructs, especially when high-level expression is driven by a foreign promoter (CMV promoter in IC2-derived constructs). In contrast, a multigene SIV DNA vaccine construct, mutated in only the *gag* nucleocapsid, was capable of producing virus particles (Gorelick et al., 2000), possibly because of more stringent control over viral protein expression via its autologous LTR promoter.

We sought to further optimize the products from these HIV-1 multigene DNA vaccine constructs by exploring alterations that would permit not only VLP assembly and budding but also core maturation and condensation. By developing the multigene constructs IC48 and IC90, which incorporate protease mutations found in some HIV-infected persons with resistance to protease inhibitors, expression in vitro resulted in the production of mature VLPs as determined by core condensation. Protease mutations at either amino acid positions 48 or 90

delay, but do not abolish, protease enzymatic activity (Jacobsen et al., 1995), unlike the D25N mutation (Kohl et al., 1988), and permit production of infectious virus when present within an otherwise wild-type provirus. Therefore, with the IC48 and IC90 vaccine constructs, aberrant intracellular protease activity was presumably diminished and VLP assembly directed by the intact Gag polyprotein precursor could occur. As is the case with wild-type HIV-1, protease functions after the budding of immature VLPs generated by IC48 or IC90 to allow for core condensation and maturation.

It was generally accepted that the main route of entry for HIV-1 is viral fusion with the plasma membrane (Hernandez et al., 1996). Maturation of the viral core appears to be an important determinant as observed for IC48, where significantly more VLP entered coreceptor-specific target cells than observed for IC25. Recently, it was suggested that the host cell via endocytosis takes up most HIV-1 particles that bind to the cell surface (Fredericksen et al., 2002; Marechal et al., 1998; Schaeffer et al., 2001). Our results indicate that immature VLP are binding to the cell surface and entering target cells by way of endocytosis regardless of the coreceptor molecule. Furthermore, IC48 VLPs appear to enter both target cells, but a greater proportion of VLP entered CCR5 cells, suggesting VLPs with matured cores may be an improvement over the immature VLP, using both the endocytic and cytosolic pathways for processing.

After budding from the muscle or other cell type in vivo that first acquires the DNA vaccine, mature VLPs potentially can enter additional distant dendritic cells. This entry mechanism would be analogous to a natural HIV infection, especially if the VLPs contain matured cores, using the cytosolic pathway for antigen processing and promoting a greater likelihood for MHC Class I and II presentation. Furthermore, these multigene DNA vaccine constructs contain a CCR5-utilizing envelope, and mature gp120 Env was detected in banded VLP preparations (Fig. 6). The HIV-1 Env protein is necessary for productive entry into target cells, and inclusion of *env* in similar DNA vaccine constructs was critical for the control of a SHIV challenge (Amara et al., 2002). Assembly of native trimeric forms of Env on the surface of mature VLPs and subsequent postfusion processing and presentation of these antigens may lead to still-unappreciated aspects of humoral immunity to challenge. Furthermore, fusion-mediated entry of mature VLPs analogous to a wild-type HIV-1 infection may generate, after subsequent antigen processing, presentation of more conserved or masked epitopes and provide better cross-subtype recognition. Preliminary in vitro experiments suggest that IC48 supernatant is capable of being recognized by persons' T cells infected with HIV of different subtypes.

In addition to producing mature VLPs, the DNA vaccine candidates IC48 and IC90 also generate HIV protein aggregates and immature VLPs. Soluble and secreted HIV-1 protein aggregates encoded by the injected DNA may be processed and presented in the context of MHC Class II molecules, stimulating B-cell immunity by generating antibodies and B-cell memory against the proteins. Immature VLPs may enter cells via phagocytosis or endocytosis, as found with other viral particles (Marechal et al., 1998; Schaeffer et al., 2001), and may also be a source of immunogens via the MHC class II pathway. Furthermore, on the surface of cells acquiring the DNA construct (muscle or dendritic antigen-presenting cells), peptides from the proteins encoded by vaccine are also presented by the MHC Class I pathway, after the proteins are processed as endogenous antigens. Resulting immunogens are expressed in a native conformation; thus, there may be an effective stimulation of CD8+ CTL and MHC Class I-restricted pathway presentation of the antigens. Cellular immunity has an important role in the control of immunodeficiency virus infections (Goulder et al., 1999) and protection against challenge with HIV (Amara et al., 2001; Barouch et al., 2000; McMichael and Phillips, 1997; Oldstone 1997; Rowland-Jones et al., 1997). Evidence further suggests that cytotoxic T-lymphocyte cross-reactivity will be an important component of a single efficacious HIV vaccine (Buseyne et al., 1998; Cao et al., 1997; Ferrari et al., 1997; McAdam et al., 1998; Rowland-Jones et al., 1999).

In a macaque model of retroviral challenge, control of viremia that correlated with protection from disease was observed with a SHIV DNA vaccine, similar in composition to those described here, and MVA boost (Amara et al., 2001). We have determined that the SHIV DNA vaccine construct forms aggregates in vitro (data not shown), as expected based on wild-type protease (similar to IC2). Furthermore, in a recent nonhuman primate immunogenicity trial evaluating HIV-1 subtype B vaccines, DNA constructs that produce only immature VLPs (with protease mutation at amino acid 25) showed enhanced ability to stimulate an immune response, especially humoral immunity, when compared with aggregate-forming DNA constructs (H. Robinson, personal communication). Therefore, the development and characterization of HIV-1 DNA vaccines that generate a balance of these different antigenic forms may provide distinct immunologic advantages and may further advance vaccine development. In addition, production of mature virus-like particles, which may behave similarly to attenuated live-virus vaccines but without risk of integration or causing AIDS, is clearly an obtainable goal for multigene HIV-1 DNA vaccine approaches. It remains to be determined from the outcome of ongoing nonhuman primate trials, testing IC25 and IC48 immunogenicity, whether these vaccine constructs, especially when coupled with an MVA-based boost immunization, will provide the unique immunologic advantages for specific and cross-subtype cellular and humoral responses as suggested by their unique in vitro characteristics.

Materials and methods

DNA immunogens

HIV-1 was obtained from a female sex worker in Abidjan, Côte d'Ivoire. An incident recombinant HIV-1 subtype A/G (CRF02_AG) strain was chosen (Ellenberger et al., 2002). HIV-1-specific reverse transcriptase (RT)-PCR was done on total RNA recovered from a plasma sample. Gag (amino acids 1–496) and Pol (amino acids 1–728) (GenBank accession no., AY227361); Vpu (amino acids 1–82), Tat (amino acids 1–102), Rev (amino acids 1–84) and Env (amino acids 1–854) (GenBank accession no., AY227362) from IC0928 were reverse transcribed, and fragments produced by DNA PCR were cloned into the pGA1 expression vector (GenBank accession no., AF425297). Sense primer 1 (5′-AAGATCTATCGATGCAAGGACTCGGCTTGC-3′) and antisense primer 2 (5′-TTCCAATTGCTGTGAGAATTCTCATGCTCTTCTTGGG-3′) were used to amplify the 5′ PCR product. The 5′ PCR product encompassed the *gag*, *protease*, and *RT* genes and was cloned by introducing a stop codon and a unique *Eco*RI restriction site at 12 amino acid residues downstream of the RT and integrase junction. Sense primer 3 (5′-AAGGGGTTAAAGCTATAATAAGAA-TTCTGCA-3′) and antisense primer 4 (5′-CCTTT-GCTGCCCTATCTGATTCTTCTAGG-3′) were used to amplify the 3′ PCR product.

The 3′ PCR fragment encompassed the *vpu*, *tat*, *rev*, and *env* sequences and the splice acceptor sites necessary for proper processing and expression of their respective mRNAs. The 3′ PCR product was cloned using an *Eco*RI restriction site (88 bp upstream of the Tat start site) and introducing a stop codon and unique *Nhe*I and *Rsr*II restriction sites, truncating Env by seven amino acid residues.

The inactivating codon mutations were made using a site-directed mutagenesis kit (Stratagene, La Jolla, CA) following the manufacturer's protocol. All mutations were confirmed by sequencing. Primer pairs used for the mutagenesis were:

Sense primer C390S 928 ZN1 (5′-GCCAGAGAATAATAAAGaGcTTCAACaGcGGCAAAGAAGG-3′) and antisense primer C393S 928 ZN2 (5′-CCTTCTTTGCCgCtGTTGAAgCtCTTTATTATTCTCTGGC-3′); sense primer C411S 928 ZN3 (5′-CCTAGAAAGAGAGGCaGcTGGAAAaGcGGAAAGGAAGG-3′) and antisense primer C414S 928 ZN4 (5′-CCTTCCTTTCCgCtTTTCCAgCtGCCTCTCTTTCTAGG-3′); sense primer D185N 928 RT1 (5′-CCAATATATGaAcGATTTATATGTAGGATCTGAC-3′) and antisense primer D185N 928 RT2 (5′-GTCAGATCCTACATATAAATCgTtCATATATTGG-3′); sense primer W266T 928 RT3 (5′-GGGAAAACTAAATaccGCAAGTCAGATTTATGCAGG-3′) and antisense primer W266T 928 RT4 (5′-CCTGCATAAATCTGACTTGCggtATTTAGTTTTCCC-3′); and sense primer E478Q 928 RT5 (5′-CCCTAATTGAGACAACAAATCAAAAGACTcAgTTACATGC-3′) and antisense primer E478Q 928 RT6 (5′-GCATGTAAcTgAGTCTTTTGATTTGTTGTCTCAATTAGGG-3′).

All protease mutations were made from a *Cla*I-to-*Xmn*I fragment (1995 bp) subclone (Table 1). The Asp-to-Asn substitution at position 25 (IC25), the Gly-to-Val substitution at position 48 (IC48), and the Met-to-Leu substitution at position 90 (IC90) were created by PCR with primers containing the mutations. The PCR-amplified mutated products were cloned back into the IC1 backbone. The mutations were made using a site-directed mutagenesis kit (Stratagene) following the manufacturer's protocol. All mutations were confirmed by sequencing. Primer pairs used for the mutagenesis were:

Sense primer D25N 928 Prt1 (5′-GCCAATAGAAGCCCTATTAaACACAGGAGC-3′) and antisense primer D25N 928 Prt2 (5′-GCTCCTGTGTtTAATAGGGCTTCTATTGGC-3′); sense primer G48V 928 Prt3 (5′-CCAAAAATGATAGtGGGAATTGGAGG-3′) and antisense primer G48V 928 Prt4 (5′-CCTCCAATTCCCaCTATCATTTTTGG-3′); and sense primer M90L 928 Prt5 (5′-GGACGAAATATGaTGACTCAGATTGGT-3′) and antisense primer M90L 928 Prt6 (5′-ACCAATCTGAGTCAtCATATTTCGTCC-3′).

Cell lines

Human 293T cells, a human kidney-derived cell line, were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. GHOST-4 cells, expressing the human CD4 gene in combination with either CXCR4 or CCR5 (Morner et al., 1999), were obtained from the National Institute of Allergy and Infectious Diseases AIDS Research and Reference Reagent Program (Rockville, MD). GHOST-4 cell cultures were maintained in DMEM supplemented with 10% fetal bovine serum, 1 μg/ml puromycin, 100 μg/ml hygomycin B, and 500 μg/ml G418.

Transient transfections

293T cells were added at 10^6 cells per well of Costar six-well plates in 2 ml of DMEM growth medium and incubated for 24 h at 37 °C in a 5% CO_2 humidified atmosphere. After 24 h, LipofectAmine2000 reagent (Invitrogen, Carlsbad, CA) and plasmid DNA were mixed according to the manufacturer's protocol and added to each well. Supernatants were harvested 24 or 48 h following the addition of transfection reagents. For protease inhibitor experiments, saquinavir (15 or 100 nM) was added to transfection cultures immediately following the addition of plasmid DNA.

Antigen-capture assay

Assay was performed using an HIV-1 antigen-capture EIA kit (Coulter, Hialeah, FL) according to the manufacturer's instructions.

Antibodies and Env ELISA

Pooled human immunoglobulin anti-HIV (catalog no. 3957) was obtained from the National Institute of Allergy and Infectious Diseases AIDS Research and Reference Reagent Program (Rockville, MD). Affinity-purified sheep polyclonal anti-gp120 was purchased from International Enzymes, Inc. Ninety-six-well plates were coated with 100 μl of 2 μg/ml of anti-gp120 in carbonate buffer and incubated at 4°C for 18 h. Samples were added to the wells following three washes and blocking, and incubated for 1 h at room temperature. Following three washes, 100 μl/well of 1:5000 dilution of HIV-Ig was added to each well and incubated for 1 h at room temperature. Following three washes, 100 μl/well of 1:10,000 dilution of anti-human-HRP was added, and incubated for 1 h at room temperature. Then, 100 μl of substrate was added and incubated for 5 min. The reaction was stopped by adding 25 μl of 2 M H_2SO_4. The plates were read at 450 nm and data were analyzed using four-parameter analysis for a standard curve.

Antibodies and Western blot analysis

Rabbit polyclonal anti-p24 (catalog no. 4250) and goat polyclonal anti-gp160 (catalog no. 188) were obtained from the National Institute of Allergy and Infectious Diseases AIDS Research and Reference Reagent Program. Western blot analysis was performed as previously described (Sambrook et al., 1989). HIV-1 protein bands were visualized using the ECL Western blotting detection reagent (Amersham Pharmacia Biotech, Piscataway, NJ) as described by the manufacturer. Expression levels were determined using a phosphorimager.

Purification of particles

Following 24 h of transient transfection of 293T cells, culture supernatants were recovered, clarified by centrifugation at 200 × *g* × 5 min, and 1 ml was layered on top of 10–50% sucrose gradients. Gradients of 10 ml consisting of 2 ml each of 50%, 40%, 30%, 20%, and 10% sucrose solutions were added to ultraclear centrifuge tubes from bottom to top. Gradients were centrifuged at 40,000 rpm for 16 h in SW41Ti rotor. Fractions of 1 ml were recovered, top to bottom. Fraction aliquots were analyzed by HIV-1 p24-antigen capture assay.

Electron microscopy

Transfected 293T cells were fixed in the multiwell plate using 1 ml of 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 2 h at 4°C. After three washes with the same buffer, 1.0% osmium tetroxide in 0.1 M cacodylate buffer was added, incubated for 1 h, dehydrated through an ethanol series, and embedded with Eponate 12 resin. Following polymerization of the resin, the cells were sectioned en face, stained with 4% uranyl acetate in water and lead citrate, and observed on a Hitachi H-7500 transmission electron microscope.

Viral entry assay

GHOST-4 cells expressing either CXCR4 or CCR5 (Morner et al., 1999) were added at 0.6 × 10^6 cells per well of Costar six-well plates in 2 ml of GHOST growth medium and incubated for 24 h at 37°C in a 5% CO_2 humidified atmosphere. The culture medium was then replaced with 600 μl of neat culture supernatant from 293T cells, 24 h after transfection with either IC25 or IC48 (engineered into the IC1 backbone to increase the level of RNA packaging). After 1 h at 37°C, the cells were washed three times with 1 ml of PBS and then trypsin-treated [600 μl of 0.05% trypsin–EDTA (Invitrogen)] at 37°C for 25 min with intermittent careful mixing. After 600 μl of DMEM containing 10% FBS was added, the entire contents of each well were transferred to a centrifuge tube, pelleted (200 × *g* × 5 min), and washed twice with PBS. Cells were pelleted once more and RNA was extracted using an RNeasy kit (Qiagen, Chatsworth, CA), according to the manufacturer's instructions. After extraction, RNA was reconstituted in DEPC-treated water and DNAse I treated. Ten-fold dilutions of RNA were used for HIV-specific RT PCR. Thermal cycling conditions for RT-PCR were as follows: 42°C, 15 min for 1 cycle; 99°C, 5 min for 1 cycle; and 5°C, 5 min for 1 cycle. Of the 20 μl cDNA reaction, 2 μl was used for DNA PCR. Thermal cycling conditions for PCR were: 95°C, 2 min for 1 cycle; denaturation, 95°C, 30 s; annealing, 55°C, 30 s; and extension 72°C, 1 min for 35 cycles; and 72°C, 7 min for 1 cycle. The primers for Env gp41 immunodominant region amplification were as follows: RT-PCR, 41R+tail30 (5′-GAACATCGATGACAAGCTTAGGTATCGATAAAC-GACAAAGGTGAGTATCCCTGCCTAA-3′); primary PCR sense primer 40F (5′-TCTTAGGAGCAGCAG-GAAGCACTATGGG-3′) and antisense primer tail30 (5′-GAACATCGATGACAAGCTTAGGTATCGATA-3′); and nested PCR sense primer 46F (5′-ACAATTATTGTCTGG-TATAGTGCAACAGCA-3′) and antisense primer 47R (5′-TTAAACCTATCAAGCCTCCTACTATCATTA-3′). An HIV-1 *env* fragment of 460 bp was detected by ethidium bromide staining of agarose gel.

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